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Conservation of the 2,4-Diacetylphloroglucinol Biosynthesis Locus Among Fluorescent *Pseudomonas* Strains from Diverse Geographical Locations

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SUBMITTED BY

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

No information in this study is being claimed confidential on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B) or (C).

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GOOD LABORATORY PRACTICE STATEMENT

This study is from the published literature. Accordingly, the submitter is unaware if the study was conducted in accordance with Good Laboratory Practice Standards (GLPS), as specified in 40 CFR Part 160.

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Conservation of the 2,4-Diacetylphloroglucinol Biosynthesis Locus among Fluorescent *Pseudomonas* Strains from Diverse Geographic Locations

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The broad-spectrum antibiotic 2,4-diacetylphloroglucinol (PHL) is a major determinant in the biological control of a range of plant pathogens by many fluorescent *Pseudomonas* spp. A 4.8-kb chromosomal DNA region from *Pseudomonas fluorescens* Q2-87, carrying PHL biosynthetic genes, was used as a probe to determine if the PHL biosynthetic locus is conserved within PHL-producing *Pseudomonas* strains of worldwide origin. The *phl* gene probe hybridized with the genomic DNA of all 45 PHL-producing *Pseudomonas* strains tested, including well-characterized biocontrol strains from the United States and Europe and strains isolated from disease-suppressive soils from Switzerland, Washington, Italy, and Ghana. The PHL producers displayed considerable phenotypic and genotypic diversity. Two phenotypically distinct groups were detected. The first produced PHL, pyoluteorin, and hydrogen cyanide and consisted of 13 strains from almost all locations sampled in the United States, Europe, and Africa. The second produced only PHL and HCN and consisted of 32 strains from the U.S. and European soils. Analysis of restriction patterns of genomic DNA obtained after hybridization with the *phl* gene probe and cluster analysis of restriction patterns of amplified DNA coding for 16S rRNA (ARDRA) and randomly amplified polymorphic DNA (RAPD) markers indicated that the strains that produced both PHL and pyoluteorin were genetically highly similar. In contrast, there was more diversity at the genotypic level in the strains that produced PHL but not pyoluteorin. ARDRA analysis of these strains indicated two clusters which, on the basis of RAPD analysis, split into several subgroups with additional polymorphisms. In general, the occurrence of phenotypically and genotypically similar groups of PHL producers did not correlate with the geographic origin of the isolates, and highly similar strains could be isolated from diverse locations worldwide.

Phloroglucinol antibiotics are phenolic bacterial and plant metabolites with antifungal (14, 23, 31, 35, 54, 55), antibacterial (6, 23, 31, 39), phytotoxic (22, 23, 40), antiviral (51), and anthelmintic (5) activity. Of particular interest is 2,4-diacetylphloroglucinol (PHL), because of its production by fluorescent *Pseudomonas* spp. of worldwide origin, e.g., Switzerland (23, 25), the United States (17, 31, 35, 55), Ireland (11, 45), England (6), Belgium (10), and Ukraine (14, 36, 37). Many *cis*-acetylphloroglucinol-producing strains have biocontrol activity against one or more plant diseases, and PHL has been shown to be the major or sole metabolite associated with pathogen suppression (7, 59). For example, *Pseudomonas fluorescens* CHA0 was isolated from a soil in the Morens region of Switzerland that is suppressive to black root rot of tobacco, caused by *Thielaviopsis basicola* (48). Strain CHA0 produces both monoacetylphloroglucinol and PHL, as well as hydrogen cyanide, pyoluteorin (PLT), and several other bioactive compounds (56). PHL contributes to the suppression of black root rot of tobacco by CHA0 (25) and is the major determinant in the suppression by CHA0 of take-all of wheat, caused by *Gaeumannomyces graminis* var. *tritici* (23). A Phl$^-$ Tn*5* mutant of strain CHA0, CHA625, was less inhibitory of *T. basicola* and *G. graminis* var. *tritici* in vitro and less suppressive of black root rot and take-all than strain CHA0 (23, 25). Complementation of CHA625 with an 11-kb fragment from a CHA0 genomic library largely restored PHL production, fungal inhibition, and disease suppression. PHL could also be isolated from the rhizosphere of wheat colonized by the wild-type CHA0 or the complemented mutant (23). In addition, amplification of the housekeeping sigma factor (σ^{70}) in CHA0 increased production of PHL and PLT severalfold in vitro and in the rhizosphere and improved protection of cucumber against *Pythium ultimum*, *Phomopsis sclerotioides*, and *Fusarium oxysporum* f. sp. *cucumerinum* and of tobacco against *T. basicola* (32, 33, 43).

P. fluorescens Q2-87 was isolated from wheat grown in a take-all-suppressive soil from near Quincy, Wash., and suppresses take-all when applied as a seed treatment (38, 55). *Pseudomonas* sp. strain F113, isolated in Ireland from the root hairs of a mature sugar beet plant, was as effective as the fungicides Thiram and Previcur N in suppressing preemergence damping off of sugar beet caused by *Pythium ultimum* (11). Strains Q2-87 and F113 produce HCN and both monoacetylphloroglucinol and PHL (17, 44, 45). Phl$^-$ Tn*5* mutants of these two strains were significantly less suppressive of disease than were their respective parental strains, and activity was partially restored upon complementation with wild-type DNA fragments homologous to those containing Tn*5* insertions in the mutants (11, 55). The 6-kb wild-type fragment from F113 was expressed in one of eight other non-PHL-producing pseudomonads into which it was introduced; the transgenic strain produced PHL and was significantly more suppressive than F113 of *Pythium ultimum* on sugar beet (11). Two cosmid clones of 25 or 37 kb containing the PHL biosynthetic locus

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TABLE 1. Origins and biocontrol activities of *Pseudomonas* strains

Strain	Biocontrol activity[a]	Source[b]	References
Reference strains			
CHA0	Tb on tobacco, Ggt on wheat, Pu on cucumber	Tobacco, Morens, Switzerland	8, 9, 48, 56
Q2-87	Ggt on wheat	Wheat, Quincy, Wash.	38, 55
Q2-87::Tn*5-1*	Reduced activity against Ggt on wheat	Derivative of Q2-87	55
Q65c-80	Ggt on wheat	Wheat, Quincy, Wash.	17, 38
Pf-5	Pu on cucumber, Pu and Rs on cotton	Cotton, Texas	19, 20, 29, 35
F113	Pu on sugar beet	Sugar beet, Ireland	11, 45
PF	St on wheat	Wheat leaves, Oklahoma	31
PFM1	St on wheat	Derivative of PF	31
PFM2	St on wheat	Derivative of PFM1	31
2-79	Ggt on wheat	Wheat, Lind, Wash.	53, 58
Q69c-80	Ggt on wheat	Wheat, Quincy, Wash.	17, 38
Isolates from Quincy soil			
Q1-87	Ggt on wheat	Wheat	This study
Q4-87	Ggt on wheat	Wheat	This study
Q5-87	Ggt on wheat	Wheat	This study
Q6-87	Ggt on wheat	Wheat	This study
Q7-87	Ggt on wheat	Wheat	This study
Q8-87	Ggt on wheat	Wheat	This study
Q9-87	Ggt on wheat	Wheat	This study
Q12-87	Ggt on wheat	Wheat	This study
Q13-87	Ggt on wheat	Wheat	This study
Q37-87	Ggt on wheat	Wheat	This study
Q86-87	Ggt on wheat	Wheat	This study
Q88-87	Ggt on wheat	Wheat	17
Q95-87	Ggt on wheat	Wheat	This study
Q107-87	Ggt on wheat	Wheat	This study
Q112-87	Ggt on wheat	Wheat	This study
Q128-87	Ggt on wheat	Wheat	17
Q139-87	Ggt on wheat	Wheat	This study
Isolates from Morens soil			
Pf1	Tb on tobacco, Pu on cucumber	Tobacco	This study
P12	Tb on tobacco, Pu on cucumber	Tobacco	This study
CA1'B2	Pu and Ps on cucumber, Rs on cotton	Cucumber	12
*C*1A1	Pu and Ps on cucumber, Rs on cotton	Cucumber	12
CM1'A2	Pu and Ps on cucumber, Rs on cotton	Cucumber	12
TM1A3	Pu and Ps on cucumber, Rs on cotton	Tomato	12
TM1'A4	Pu and Ps on cucumber, Rs on cotton	Tomato	This study
TM1A5	Pu and Ps on cucumber, Rs on cotton	Tomato	12
TM1'A5	Pu and Ps on cucumber, Rs on cotton	Tomato	This study
TM1B2	Pu and Ps on cucumber, Rs on cotton	Tomato	12
Isolates from Alhenga soil			
PINR2	Pu on cucumber, Forl on tomato	Tobacco	This study
PINR3	Pu on cucumber, Forl on tomato	Tobacco	This study
PITR2	Pu on cucumber, Forl on tomato	Wheat	This study
PITR3	Pu on cucumber, Forl on tomato	Wheat	This study
PILH1	Pu on cucumber, Forl on tomato	Tomato	This study
Isolates from Ghanion soil			
PGNR1	Pu on cucumber, Forl on tomato	Tobacco	This study
PGNR2	Pu on cucumber, Forl on tomato	Tobacco	This study
PGNR3	Pu on cucumber, Forl on tomato	Tobacco	This study
PGNL1	Pu on cucumber, Forl on tomato	Tobacco	This study
PGNR4	Pu on cucumber, Forl on tomato	Tobacco	This study

[a] Tb, *T. basicola*; Ggt, *G. graminis* var. *tritici*; Pu, *Pythium ultimum*; Rs, *R. solani*; Ps, *Phomopsis sclerotioides*; and Forl, *F. oxysporum* f. sp. *radicis-lycopersici*; St, *Septoria tritici*. Data on biocontrol activity for most of the *Pseudomonas* strains come from unpublished experiments.

[b] With the exception of strain PF and its derivatives, which were isolated from wheat leaves, *Pseudomonas* strains were isolated from roots of indicated plants

from strain Q2-87 also were expressed in nonproducers, namely, *P. fluorescens* 2-79 and *Pseudomonas* strain 5097 (55); a subclone of approximately 6.5 kb conferred PHL biosynthesis to all strains and improved the biocontrol activity of some of 27 fluorescent *Pseudomonas* spp. into which it was introduced (16).

Our observation of the widespread occurrence of PHL-producing fluorescent *Pseudomonas* spp. with biocontrol activity prompted us to investigate if the PHL biosynthetic locus is conserved among strains of worldwide origin. Using a cloned fragment from the PHL biosynthetic region of *P. fluorescens* Q2-87 (2, 3, 16, 55; GenBank accession no. U41818) as a probe, we demonstrated that this region is conserved in all of the strains in our collection known to produce PHL. By analysis of metabolite patterns and molecular typing, we also found that there is considerable diversity among PHL-producing pseudomonads in soils originating from different geographic locations.

TABLE 2. Biological and physical properties of disease-suppressive soils used in this study

Designation	Country of origin	Suppressive effect	Type	% (wt/wt)				pH	Reference(s)
				Organic matter	Sand	Silt	Clay		
Morens soil (MS1)	Switzerland	Black root rot of tobacco	Sandy loam	2.4	46	39	15	6.2	48–50
Quincy soil	United States	Take-all of wheat	Silt loam	1.1	35	52	13	5.6	38
Albenga soil	Italy	*Fusarium* wilt of tomato	Sandy loam	2.3	58	29	13	7.0	52
Ghana soil	Ghana	*Fusarium* wilt of tomato	Silt loam	1.2	38	48	14	6.5	International Institute of Tropical Agriculture, Ibadan, Nigeria

MATERIALS AND METHODS

Isolation of bacteria from suppressive soils and assessment of biocontrol activity. *Pseudomonas* strains used in this work are listed in Table 1. The reference strains are well-characterized biological control agents. The other *Pseudomonas* strains listed in Table 1 were isolated from disease-suppressive soils described in Table 2. The characteristics of soil from Morens, Switzerland, suppressive to tobacco black root rot, and soil from Albenga, Italy, suppressive to *Fusarium* wilt, have been described in detail previously (48–50, 52). Strains from the take-all-suppressive soil from near Quincy, Wash., were isolated as described by Pierson and Weller (38). To isolate bacteria from the other suppressive soils, surface-sterilized seeds of cucumber (*Cucumis sativus* cv. Chinesische Schlange), tobacco (*Nicotiana glutinosa*), tomato (*Lycopersicon esculentum* cv. Bonny Best), and wheat (*Triticum aestivum* cv. Arina) (23, 24) were sown in pots containing 400 ml of soil. After 4 to 6 weeks of growth in a growth chamber at 70% relative humidity with 16 h of light at 22°C and 8 h of dark at 18°C, the adhering soil was gently removed from the roots. The roots were washed in double-distilled sterile water by vigorous agitation on a rotary shaker. For isolation of bacteria from the root interior, the roots were then subjected to treatment with 70% ethanol for 10 s followed by 5% H_2O_2 for 10 min and rinsed three times with double-distilled sterile water. Roots were cut into 0.5- to 1-cm pieces and placed on S1 medium, which is selective for fluorescent pseudomonads (15), or on King's medium B agar (KMB [27]). After incubation for 2 to 3 days at 24°C, bacterial colonies that developed along the root pieces were transferred to fresh KMB plates and purified by repeated subculturing. The isolates were then tested for biocontrol activity (Table 1) against take-all of wheat caused by *G. graminis* var. *tritici*, black root rot of tobacco caused by *T. basicola*, black root rot of cucumber caused by *Phomopsis sclerotioides*, and crown and root rot of tomato caused by *F. oxysporum* f. sp. *radicis-lycopersici* as described elsewhere (12, 17, 30, 48, 55). The ability of the bacterial isolates to suppress damping-off of cucumber caused by *Pythium ultimum* and of cotton caused by *Rhizoctonia solani* was tested as follows. Plastic pots (160 ml) were filled with natural soil, and a millet seed inoculum (32, 33) of *Pythium ultimum* 67-1 (obtained from Allelix Agriculture, Mississauga, Canada; 4 g of inoculum per liter of soil) or of *R. solani* 260 (obtained from Ciba Ltd., Basel, Switzerland; two overgrown millet seeds per pot) was mixed into the soil. Two hours later, the bacterial suspensions were mixed into soil to give 10^7 CFU/cm^3. Then, four 2-day-old seedlings of, respectively, cucumber and cotton (*Gossypium hursutum* cv. Stoneville 506, obtained from Ciba) were planted per pot. After 5 days (cotton) or 12 days (cucumber) of incubation in a growth chamber under the conditions described above, the number of emerged plants per pot was counted.

Bacteria, plasmids, fungi, and culture conditions. *Pseudomonas* strains were cultivated in Luria-Bertani (LB) broth (42) or on KMB at 24°C. *Escherichia coli* strains were grown in LB broth or on nutrient agar (47) at 37°C. *E. coli* HB101 (4) was used as the host for plasmids pMON5123 and pMON5118, each containing genes involved in the biosynthesis of PHL (2, 3, 55; GenBank accession no. U41818). When appropriate, tetracycline hydrochloride and kanamycin sulfate were used as selective antibiotics at 50 μg/ml. *G. graminis* (Sacc.) Arx & Olivier var. *tritici* Walker strain FSLN-1 was routinely cultivated on potato dextrose agar (Difco) at 24°C.

Extraction and detection of metabolites. Production of HCN was detected by growing *Pseudomonas* strains for 16 h at 24°C in 100 μl of LB broth in wells of microtiter dishes with an indicator paper tightly covering the wells as described by Voisard et al. (57). Production of a fluorescent siderophore(s) was detected by growing the strains for 16 h at 24°C on KMB or succinate minimal medium (34), with or without addition of 100 μg of EDDHA [ethylenediamine-di(*o*-hydroxyphenylacetic acid)] per ml, and observing the fluorescence of the colonies under UV light (at a wavelength of 366 nm; Omnilab, Mettmenstetten, Switzerland). Production of the antibiotics PHL, monoacetylphloroglucinol, and PLT was determined by growing strains on malt agar (15 g of Difco malt extract, 17 g of Difco agar, and 1 liter of double-distilled water, adjusted to pH 7 with 1 N NaOH) or KMB at 24°C for 64 h. The agar media plus the bacteria were extracted with acetone and ethyl acetate, and the extract was analyzed by reverse-phase high-performance liquid chromatography as described previously (23, 33). Isolation of the antibiotics was done twice.

Fungal inhibition assay. Inhibition of *G. graminis* var. *tritici* by the *Pseudomonas* strains on malt agar and KMB plates was performed essentially as described by Thomashow and Weller (53). Bacteria were grown overnight in LB broth, and 5 μl of each culture was spotted 0.5 cm from the edge of the plate (four spots per plate). One hour later, a 0.6-cm circular plug from a culture of *G. graminis* var. *tritici* was assessed after 7 days by measuring the distance between the edges of the bacterial colony and the fungal mycelium. Inhibition was expressed as the percentage of inhibition caused by strain CHA0 that had been spotted on the same plate. Assays were repeated at least twice, with three replicate plates per treatment.

DNA extraction and Southern hybridization. Chromosomal DNA was isolated as described by Gamper et al. (13). Plasmid DNA was prepared with a plasmid midi kit (QIAGEN Inc., Hilden, Germany). Restriction enzyme digestions (Boehringer Mannheim Biochemicals), agarose gel electrophoresis, DNA fragment isolation from low-melting-point agarose, oligolabelling (Pharmacia, Uppsala, Sweden), and Southern transfer to Hybond-N nylon membranes (Amersham) were performed by standard methods (42) or as recommended by the suppliers.

ARDRA. The DNA coding for the 16S rRNA of the *Pseudomonas* strains was amplified with primers R1n and 1488r (Table 3; sequences kindly provided by R. Simon and H.-V. Tichy, TÜV Südwest, Freiburg, Germany; primers synthesized by MWG Biotech, Basel, Switzerland). *E. coli* K-12 and *P. aeruginosa* PAO1 (28) were used as controls. Bacterial strains were grown for 24 h at 27°C in 150 μl of 1/10 strength KMB broth in wells of microtiter plates. Aliquots (0.5 μl) of each bacterial culture were transferred to PCR tubes containing 4.5 μl of lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.1% Tween 20) and were then heat lysed (10 min at 99°C). PCR amplification was carried out in 20-μl reaction mixtures, each containing 5 μl of heat-lysed bacterial culture, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 1% Triton X-100, 100 μM (each) dATP, dCTP, dGTP, and dTTP (Stehelin Cie., Basel, Switzerland), 0.28 μM primer, and 0.15 U of *Taq* DNA polymerase (Stehelin Cie.). Amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR System 9600. The PCR cycling program consisted of an initial denaturation at 94°C for 120 s followed by 35 cycles of 94°C for 40 s, 50°C for 60 s, and 72°C for 60 s, and the cycling was followed by a final extension at 72°C for 180 s. Restriction of 5 μl of the amplified product was performed in a total volume of 20 μl of restriction buffer (Boehringer) with 1.5 U of either *Cfo*I, *Hin*fI, *Taq*I, *Alu*I, *Rsa*I, *Hpa*II, *Hae*III, or *Dde*I (Boehringer). The restricted DNA was then electrophoresed in a 2.5% agarose gel with 1×

TABLE 3. Oligonucleotides used for ARDRA and PCR-RAPD analysis

Primer	Sequence	Length (bases)	G+C content (%)	Evaluation technique
R1n	GCTCAGATTGAACGCTGGCG	20	60	ARDRA
1488r	CGGTTACCTTGTTACGACTTCACC	24	50	ARDRA
D7	TTGGCACGGG	10	70	PCR-RAPD
GAC	CCGTTATTGCGCCCGG	16	69	PCR-RAPD
M12	GGGACGTTGG	10	70	PCR-RAPD
M13	GGTGGTCAAG	10	60	PCR-RAPD

TABLE 4. Phenotypic characteristics and in vitro antifungal activities of *Pseudomonas* strains isolated from soils of different geographic origins

Strain	Production[a] of:							Relative inhibition[b] of Ggt on:	
	HCN	Siderophores	PHL on MA	PLT on MA	PHL on KMB	PLT on KMB	Red P. on KMB	MA	KMB
Reference strains									
CHA0	+	++	2.5	BD	BD	14.0	+	1.00	1.00
Q2-87	+	+	35.6	BD	37.4	BD	+++	1.72	1.28
Q2-87::Tn*5-1*	+	+ -	BD	BD	BD	BD	-	0.13	0.79
Q65c-80	-	-	14.4	BD	35.4	BD	+++	1.59	1.28
Pf-5	+	+ -	9.2	4.2	BD	127.7	++	1.50	1.48
F113	+	+	11.8	BD	58.7	BD	+++	1.27	0.96
PF	-	+ -	1.3	BD	12.8	30.0	++ -	1.44	1.27
PFM1	+	++	3.4	BD	11.2	3.7	+ -	1.44	1.30
PFM2	-	+ -	12.2	6.4	2.3	73.5	+	1.33	1.34
2-79 (PCA^+)[c]	-	+++	BD	BD	BD	BD	-	0.76	0.87
Q69c-80	+	++ -	BD	BD	BD	BD	-	0.12	0.85
Isolates from Quincy soil									
Q1-87	+	+	13.9	BD	18.4	BD	+++	1.33	1.17
Q4-87	-	+	8.5	BD	16.1	BD	++ -	1.25	1.24
Q5-87	+	+	22.7	BD	11.2	BD	++ -	1.13	1.08
Q6-87	-	-	19.1	BD	20.8	BD	+++	1.18	0.96
Q7-87	-	+	15.4	BD	2.5	BD	++ -	1.24	0.80
Q8-87	+	-	5.2	BD	8.3	BD	+ - +	1.14	0.86
Q9-87	-	-	15.0	BD	13.5	BD	++ -	1.08	0.86
Q12-87	+	+	11.8	BD	14.4	BD	++ -	1.28	0.96
Q13-87	-	-	15.3	BD	13.1	BD	+ - +	1.38	1.08
Q37-87	-	+	18.2	BD	11.9	BD	+ + -	1.23	1.04
Q86-87	+	-	30.6	BD	4.9	BD	+ - -	1.19	1.26
Q88-87	-	-	24.5	BD	13.1	BD	+++	1.19	1.04
Q95-87	-	+	30.7	BD	14.8	BD	- + -	1.19	0.91
Q107-87	+	-	27.8	BD	37.9	BD	+ - +	1.04	0.83
Q112-87	-	-	4.9	BD	45.3	BD	- + -	1.07	1.00
Q128-87	+	+	18.9	BD	27.7	BD	+ - -	1.09	1.17
Q139-87	-	-	20.4	BD	26.4	BD	+++	1.07	0.96
Isolates from Morens soil									
Pf1	+	++	1.4	0.1	BD	23.9	+	1.39	1.74
P12	-	-	12.8	BD	9.8	BD	+++	1.48	1.26
CΔ1'B2	+	+	24.3	BD	24.5	BD	+ - +	1.27	1.08
C"1A1	+	-	63.5	BD	16.4	BD	+ - +	1.30	1.12
CM'1A2	-	-	27.7	BD	15.2	BD	- + +	1.36	1.12
TM1A3	+	-	25.0	BD	36.4	BD	+ + -	1.20	1.05
TM1'A4	+	+	30.6	BD	26.0	BD	+ - +	1.31	0.95
TM1A5	-	-	30.8	BD	14.2	BD	- + -	1.31	1.18
TM1'A5	+	+	31.1	BD	40.7	BD	+ + -	0.98	1.05
TM1B2	-	-	25.4	BD	18.0	BD	+ + +	1.22	1.14
Isolates from Albenga soil									
PINR2	+	++	4.5	BD	BD	12.4	+	1.30	1.16
PINR3	-	- -	2.3	BD	BD	3.2	-	1.31	1.07
PITR2	+	+	37.3	BD	48.3	BD	+ + -	1.46	1.10
PITR3	+	-	29.0	BD	47.4	BD	+ - +	1.40	0.90
PILH1	-	+	19.6	BD	35.2	BD	- + -	1.42	1.12
Isolates from Ghana soil									
PGNR1	+	++	3.6	BD	BD	9.9	+	1.62	1.15
PGNR2	-	+ -	3.9	BD	BD	10.2	-	1.65	1.15
PGNR3	+	++	6.1	BD	BD	7.8	+	1.20	1.32
PGNL1	+	++	2.4	BD	BD	3.2	+	1.17	1.46
PGNR4	-	+ -	4.4	BD	BD	4.7	+	1.29	1.43
			(6.7)[d]	(1.3)[d]	(8.1)[d]	(3.7)[d]		(0.18)[d]	(0.22)[d]

[a] Production of HCN and an unidentified red pigment (Red P.) by the bacteria was detected on KMB after 16 h and 4 to 7 days of growth at 24°C, respectively; production of fluorescent siderophores was recorded on KMB and on minimal medium succinate with or without EDDHA after 16 h of incubation at 24°C. Symbols: -, no; +, little; ++, strong; and +++, abundant production of the respective metabolite. Production of PHL and PLT (in micrograms per milliliter of malt agar [MA] or KMB). BD, below detection limit, i.e., <0.08 μg/ml for PHL and <0.1 μg/ml for PLT. Each PHL-producing strain also produced monoacetylphloroglucinol. The bacteria were grown for 64 h at 24°C. After this time, bacterial numbers per plate were not significantly different and varied between 1.1×10^{11} and 1.4×10^{11} CFU on KMB and between 0.9×10^{11} and 1.3×10^{11} CFU on malt agar. The plates were extracted as described in Materials and Methods. Each value is the mean of two replicate determinations.

[b] Inhibition of the growth of *G. graminis* var. *tritici* (Ggt) on malt agar (MA) and KMB at 24°C was measured after 7 days and is expressed relative to the inhibition by strain CHA0. Each value is the mean of at least two assays with three replicate plates per treatment.

[c] Strain 2-79 produces phenazine-1-carboxylic acid (PCA) on malt agar and KMB.

[d] Following a significant *F* test, a least-significant-difference analysis ($P = 0.05$) was performed. At the bottom of each column containing numbers, the least significant difference values for two assays (antibiotic production) and for three assays (inhibition of *G. graminis* var. *tritici*) are listed in parentheses.

TBE (90 mM Tris borate, 2 mM EDTA [pH 8.3]), stained with ethidium bromide, and photographed under UV light. The 100-bp DNA ladder (GIBCO BRL Life Technologies, Inc., Gaithersburg, Md.) was used as molecular size marker. Analysis of amplified ribosomal DNA restriction analysis (ARDRA) products obtained from extracted DNA gave the same results as analysis of ARDRA products from lysed cells (data not shown).

PCR-RAPD analysis. For the further genotypic characterization of the *Pseudomonas* strains, a PCR-based fingerprinting method with randomly amplified polymorphic DNA (RAPD) markers was applied, using a modification of the method described by Williams et al. (60). Sixty-four primers were screened, and four primers that produced a distinct and consistent banding pattern with polymorphic markers were selected (Table 3) and further evaluated. Primers D7, M12, and M13 were obtained from a series of random oligonucleotides (Operon Technologies Inc., Alameda, Calif.), and primer GAC was synthesized by the Institute of Microbiology, Swiss Federal Institute of Technology, Zurich, Switzerland; the nucleotide sequences and G+C contents of the primers are listed in Table 3. Bacterial cells were grown and lysed as described above for ARDRA. Amplification conditions were modified as follows: the reaction volume was 15 μl, the $MgCl_2$ concentration was 2 mM, and the PCR cycling program consisted of 2 cycles of 94°C for 30 s, 36°C for 30 s, and 72°C for 120 s; 20 cycles of 94°C for 20 s, 36°C for 15 s, 45°C for 15 s, and 72°C for 90 s; 19 cycles of 94°C for 20 s (incubation time increased 1 s per cycle) 36°C for 15 s, and 45°C for 15 s, and 72°C for 120 s, (incubation time increased 3 s per cycle); and a final incubation at 72°C for 10 min (28). The amplification products were electrophoresed in a 1.5% agarose gel with 1× TBE. Analysis of PCR products obtained from extracted DNA gave the same results as analysis of PCR products from lysed cells (data not shown).

Calculation of similarity coefficients and similarity analysis. All PCR-RAPD reactions were repeated at least three times, and ARDRA reactions were carried out once. RAPD fingerprints were compared only for strains showing identical patterns in ARDRA, and only the RAPD bands which appeared consistently were evaluated. Calculation of the pairwise coefficients of similarity (21), based on the presence or absence of bands, and cluster analysis with the unweighted pair group method with arithmetic mean (UPGMA) algorithm were performed with the NTSYS-pc numerical taxonomy and multivariate analysis system (41).

RESULTS

Metabolite patterns. Ten of the 11 reference *Pseudomonas* strains and all 37 of the strains originating from suppressive soils produced HCN and fluorescent siderophores; strain 2-79, which produces phenazine-1-carboxylic acid, did not produce HCN (Table 4). On the basis of the metabolites produced, there were two phenotypically distinct groups of PHL producers. One consisted of strains that produced PHL but not PLT; it included all the strains from the Quincy, Wash., soil; most of the strains from the Morens, Switzerland, soil; some strains from the Albenga, Italy, soil; and strain F113 from Ireland (Table 4). The second consisted of strains that produced both PHL and PLT; it included CHA0, Pf1, and P12 from the Swiss soil; Pf-5 and PF from U.S. soils; strains from the Ghanian soil; and two strains from the Italian soil (Table 4).

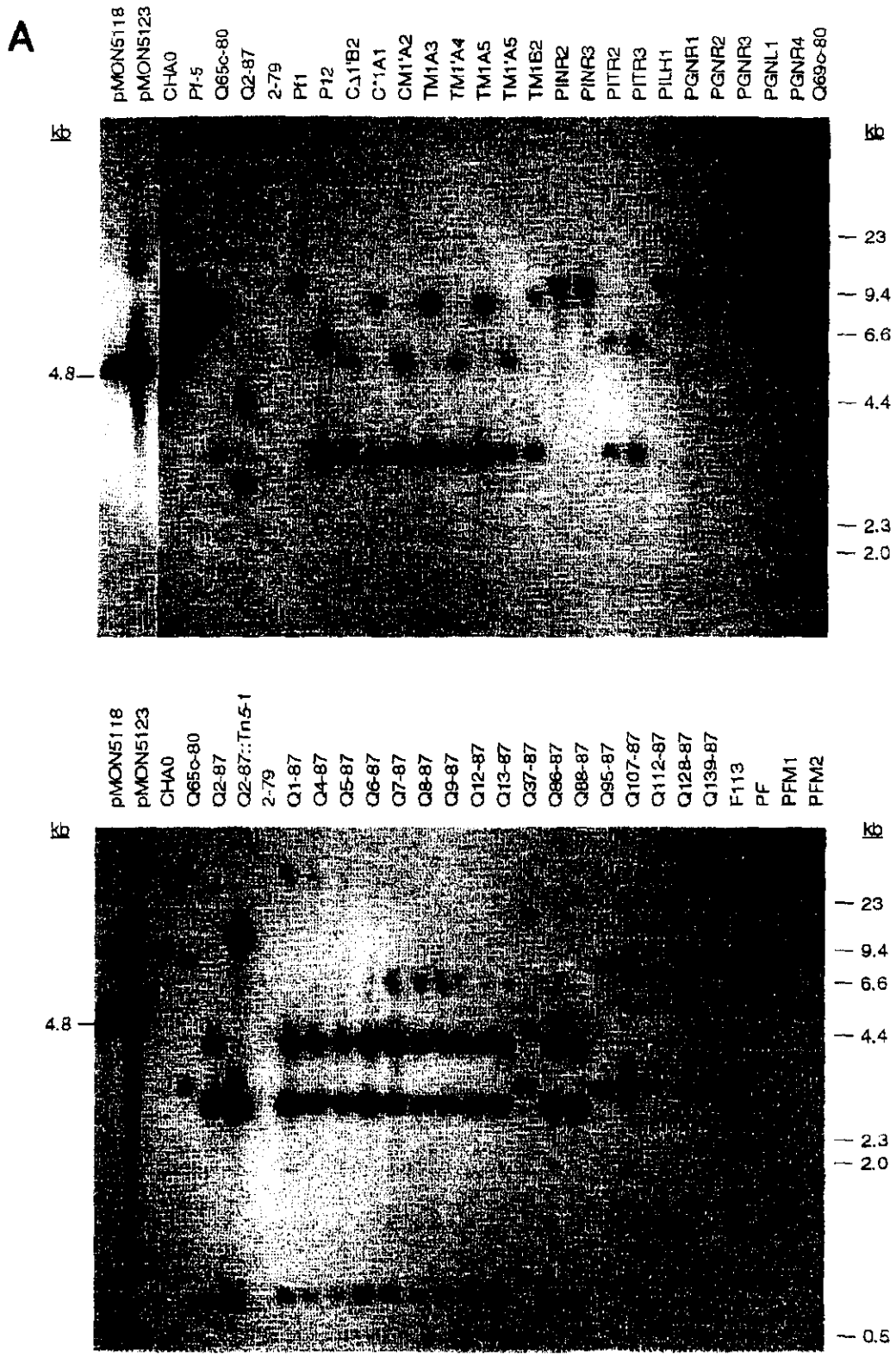
There was considerable variation in the amount of PHL and PLT produced by the different *Pseudomonas* strains. The amounts of the two antibiotics recovered ranged from 1.3 to 63.5 μg/ml for PHL and from 0.1 to 127.7 μg/ml for PLT (Table 4). In addition, each PLT-producing strain produced monoacetylphloroglucinol (data not shown) and, after 4 to 7 days of incubation on KMB, an unidentified red pigment, which is not PHL (Table 4). A Tn*5-1* Phl^- mutant of Q2-87 and the naturally PHL-negative strains 2-79 and Q69c-80, which were used as controls, did not produce the red pigment.

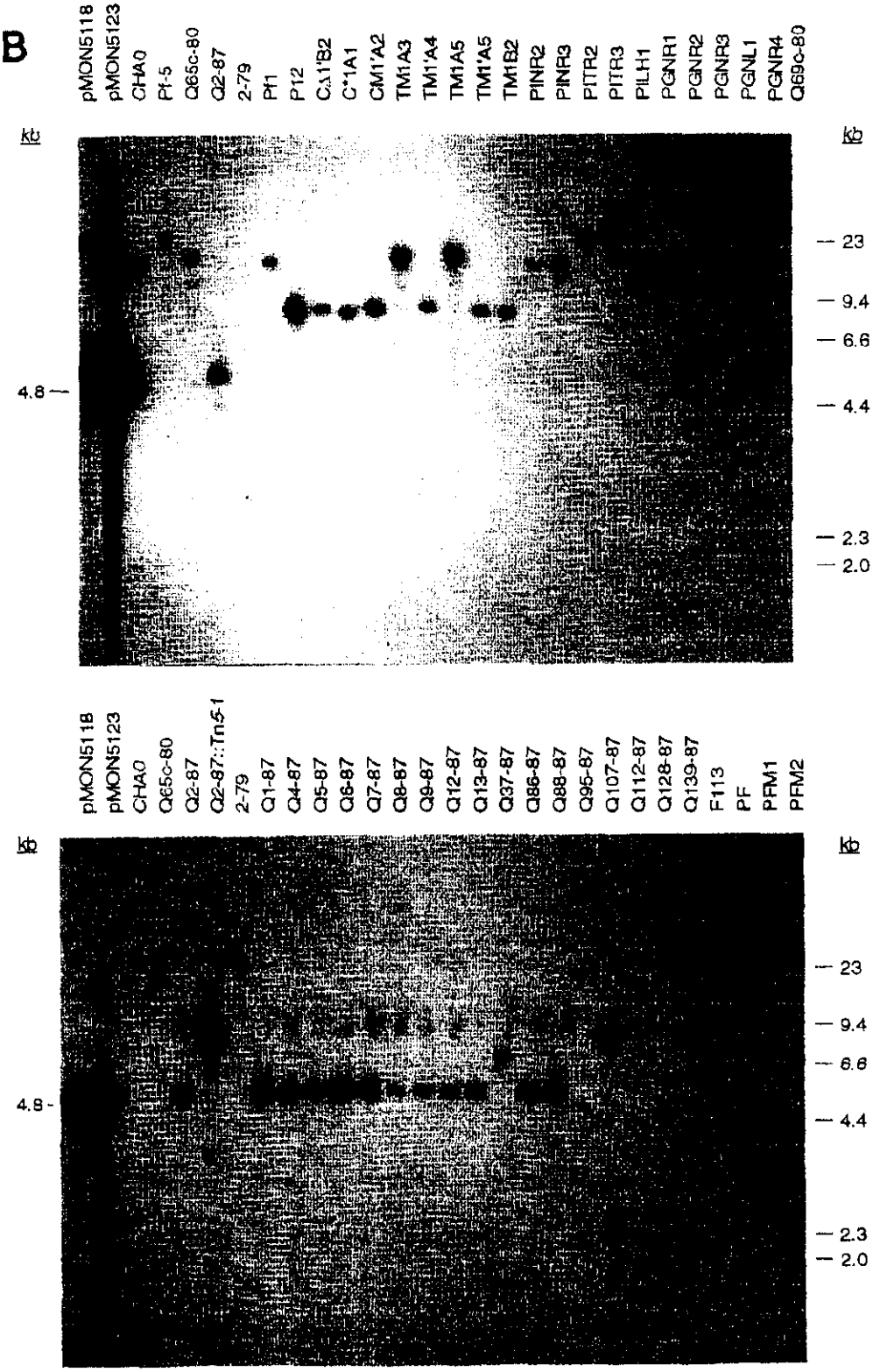
Antifungal activity. In vitro inhibition of *G. graminis* var. *tritici* by the *Pseudomonas* strains on malt agar and KMB was compared with that by *P. fluorescens* CHA0. On malt agar plates, most of the PHL-producing strains were more inhibitory of *G. graminis* var. *tritici* than strain CHA0 was (Table 4). On KMB, the inhibition by most of the strains varied in a range of about ±15% of the inhibition obtained with strain CHA0. On malt agar, the PHL-negative control strains, namely, Q2-87::Tn*5-1*, Q69c-80, and 2-79, were significantly less inhibitory than the PHL-producing strains (Table 4). In the case of strain 2-79, the relatively high inhibitory activity against *G. graminis* var. *tritici* may be attributed to the antibiotic phenazine-1-carboxylic acid, which is produced on malt agar and KMB (Table 4). The considerable index of inhibition produced by strains Q2-87::Tn*5-1* and Q69c-80 on KMB may be attributed to the fact that both strains still produce a siderophore(s) and HCN on this medium (Table 4).

Southern hybridization with the *phl* gene probe. The 4.8-kb *Bam*HI fragment carrying PHL biosynthetic genes from strain Q2-87 hybridized with the genomic DNA of each of the 45 PHL-producing *Pseudomonas* strains and of Q2-87::Tn*5-1* but not with the DNA of the naturally PHL-negative 2-79 and Q69c-80 controls (Fig. 1). Analysis of restriction fragment length polymorphisms (RFLPs) generated by digestions of DNA from the 45 PHL producers with *Eco*RV indicated five major groups (Table 5). One group included *Pseudomonas* strains from Swiss, Italian, Ghanian, and U.S. soils which had restriction patterns identical to that of reference strain CHA0 and which produced PLT in addition to PHL (Fig. 1A; Table 5); strain PILH1 was an exception because it produced no PLT. The other four groups consisted of strains that produced PHL but not PLT, including a group of similar Swiss and U.S. strains, a group of U.S. strains isolated from the Quincy soil having patterns identical to the reference strain Q2-87, and two groups consisting of strains from Italian and Swiss soils, respectively (Fig. 1; Table 5). Two strains, Q37-87, isolated from the Quincy soil, and F113, isolated from soil in Ireland, did not fit into any of the other groups. Restriction fragment patterns generated by digestion with *Bam*HI yielded more polymorphisms and a further splitting of the above RFLP groups obtained after restriction with *Eco*RV (Fig. 1B; Table 5). However, most of the strains that produced both PHL and PLT, as well as the PHL producers from the Quincy soil, which are highly similar to the reference strain Q2-87, were contained within the same groups as described above (Fig. 1B; Table 5). With the *Bam*HI digestion, Q37-87 and F113 again were distinct from the other strains.

ARDRA fingerprints. Results of ARDRA indicated three groups of PHL-producing strains (Fig. 2; Table 5). Group 1 contained all strains that produced PHL and PLT, including strains from Swiss (e.g., CHA0), U.S. (e.g., Pf-5), Italian, and Ghanian soils. These strains clustered with the other PHL-producing strains at a similarity of 0.63, indicating quite a loose relationship (Fig. 2). With the exception of strains F113 and P12, which form a separate group (group 3), all of the other strains that produced PHL but not PLT gave identical ARDRA fingerprints and were contained within group 2 (Fig. 2; Table 5). Each of these two groups included one of the naturally PHL-negative control strains; i.e., strain Q69c-80 belonged to group 2, and strain 2-79 clustered with group 3 (Fig. 2).

FIG. 1. Southern hybridization of the *phl* genomic region in *Pseudomonas* strains. Chromosomal DNA (~1 to 3 μg) from PHL-producing reference strains and isolates from suppressive soil from Morens, Switzerland; Quincy, Wash.; Albenga, Italy; and Ghana was digested with *Eco*RV (A) or *Bam*HI (B). The DNA of the recombinant plasmids pMON5118 and pMON5123 was digested with *Bam*HI. The digested DNA was electrophoresed in a 0.7% agarose gel (42 V for 16 h), transferred to a Hybond nylon membrane (Amersham), and hybridized under conditions of high stringency to the 4.8-kb *Bam*HI fragment of pMON5123 (3, 55) as specified by Sambrook et al. (42) or the manufacturers.


A
pMON5118
pMON5123
CHA0
Pf-5
Q65c-80
Q2-87
2-79
Pf1
P12
CΔ1'B2
C'1A1
CM1'A2
TM1A3
TM1'A4
TM1A5
TM1'A5
TM1B2
PINR2
PINR3
PITR2
PITR3
PILH1
PGNR1
PGNR2
PGNR3
PGNL1
PGNR4
Q69c-80
kb
4.8
23
9.4
6.6
4.4
2.3
2.0
pMON5118
pMON5123
CHA0
Q65c-80
Q2-87
Q2-87::Tn5-1
2-79
Q1-87
Q4-87
Q5-87
Q6-87
Q7-87
Q8-87
Q9-87
Q12-87
Q13-87
Q37-87
Q86-87
Q88-87
Q95-87
Q107-87
Q112-87
Q128-87
Q139-87
F113
PF
PFM1
PFM2
kb
4.8
23
9.4
6.6
4.4
2.3
2.0
0.5


B
pMON5118
pMON5123
CHA0
Pf-5
Q65c-80
Q2-87
2-79
Pf1
P12
CΔ1'B2
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CM1'A2
TM1A3
TM1'A4
TM1A5
TM1'A5
TM1B2
PINR2
PINR3
PITR2
PITR3
PILH1
PGNR1
PGNR2
PGNR3
PGNL1
PGNR4
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pMON5118
pMON5123
CHA0
Q65c-80
Q2-87
Q2-87::Tn5-1
2-79
Q1-87
Q4-87
Q5-87
Q6-87
Q7-87
Q8-87
Q9-87
Q12-87
Q13-87
Q37-87
Q86-87
Q88-87
Q95-87
Q107-87
Q112-87
Q128-87
Q139-87
F113
PF
PFM1
PFM2
kb
4.8
23
9.4
6.6
4.4
2.3
2.0

TABLE 5. Genotypic and phenotypic similarities of PHL-producing *Pseudomonas* biocontrol strains originating from soils from different geographic locations worldwide

Strain	*Eco*RV RFLP group[a]	*Bam*HI RFLP group[a]	ARDRA group[b]	RAPD group (similarity of ≥50%)[c]	Production of PLT[d]	Origin[e]
CHA0	1	1	1	1	+	Switzerland
Pf-5	1	2	1	2	+	Texas
PF	1	1	1	1	+	Switzerland
PINR2	1	1	1	1	+	Italy
PINR3	1	1	1	1	+	Italy
PILH1	1	1	2	5	+	Italy
PGNR1	1	1	1	1	+	Ghana
PGNR2	1	1	1	1	+	Ghana
PGNR3	1	1	1	1	+	Ghana
PGNL1	1	1	1	1	+	Ghana
PGNR4	1	1	1	1	+	Ghana
PF	1	2	1	2	+	Oklahoma
PFM1	1	2	1	2	+	Oklahoma
PFM2	1	2	1	2	+	Oklahoma
Q65c-80	2	3	2	3	−	Washington
Q95-87	2	3	2	3	−	Washington
Q107-87	2	8	2	3	−	Washington
Q112-87	2	8	2	3	−	Washington
Q128-87	2	8	2	3	−	Washington
Q139-87	2	8	2	3	−	Washington
CA1A1	2	5	2	3	−	Switzerland
TM1A3	2	3	2	3	−	Switzerland
TM1A5	2	3	2	3	−	Switzerland
TM1B2	2	5	2	3	−	Switzerland
Q2-87	2	4	2	4	−	Washington
Q1-87	3	4	2	4	−	Washington
Q4-87	3	4	2	4	−	Washington
Q5-87	3	4	2	4	−	Washington
Q6-87	3	4	2	4	−	Washington
Q7-87	3	4	2	4	−	Washington
Q8-87	3	4	2	4	−	Washington
Q9-87	3	4	2	4	−	Washington
Q12-87	3	4	2	4	−	Washington
Q13-87	3	4	2	4	−	Washington
Q86-87	3	4	2	4	−	Washington
Q88-87	3	4	2	4	−	Washington
P12	3	5	3	8	−	Switzerland
PITR2	4	6	2	5	−	Italy
PITR3	4	6	2	5	−	Italy
CA1'B2	4	5	2	3	−	Switzerland
CM1'A2	5	5	2	3	−	Switzerland
TM1'A4	5	5	2	3	−	Switzerland
TM1'A5	5	5	2	3	−	Switzerland
Q37-87	6	7	2	6	−	Washington
F113	7	9	3	7	−	Ireland

[a] RFLPs based on digestion of total genomic DNA with *EcoRV* or *BamHI* and hybridization to a 4.8-kb probe from the PHL biosynthetic region of *Pseudomonas* strain Q2-87 (2, 3, 55) (Fig. 1).

[b] ARDRA groups of similar restriction patterns generated with eight selected restriction endonucleases were determined by cluster analysis with Jaccard's similarity coefficient and the UPGMA clustering algorithm (Fig. 3).

[c] RAPD data generated with four selected arbitrary primers were assigned to groups of >50% similarity based on a cluster analysis with Jaccard's similarity coefficient and the UPGMA clustering algorithm (Fig. 4).

[d] +, production; −, no production of PLT on KMB.

[e] For more details, see Table 1.

RAPD fingerprints. RAPD fingerprints were generated with four primers, and one example is shown in Fig. 3. Each of the selected primers produced about three or four bands. ARDRA group 1 (see above), which included strains that produced PHL and PLT, consisted of two very homogeneous subgroups with respect to their RAPD fingerprints. In the first subgroup, strains CHA0, Pf1, PINR2, PINR3, PGNR1, PGNR2, PGNR3, PGNL1, and PGNR4, isolated from Swiss, Italian, and Ghanian soils, shared the same subset of the 14 markers evaluated (Fig. 3; Table 4). The second subgroup included strains Pf-5, PF, PFM1, and PFM2, isolated from U.S. soil, with identical RAPD fingerprints (Fig. 3; Table 4); these strains clustered with the strains isolated from the European and from the Ghanian soil at a similarity of 0.50. The large ARDRA group 2 (see above), consisting of strains that produced PHL but not PLT, split into three major subgroups that showed a higher degree of polymorphism (Fig. 4; Table 4); one subgroup included very similar strains (Q65c-80, Q95-87, Q107-87, Q128-87, Q139-87, TM1A3, TM1A5, TM1B2, CA1'B2, CM1'A2, TM1'A4, and TM1'A5) from U.S. and Swiss soils, another subgroup consisted of strains similar to Q2-87 that were isolated from Quincy soil, and another sub-

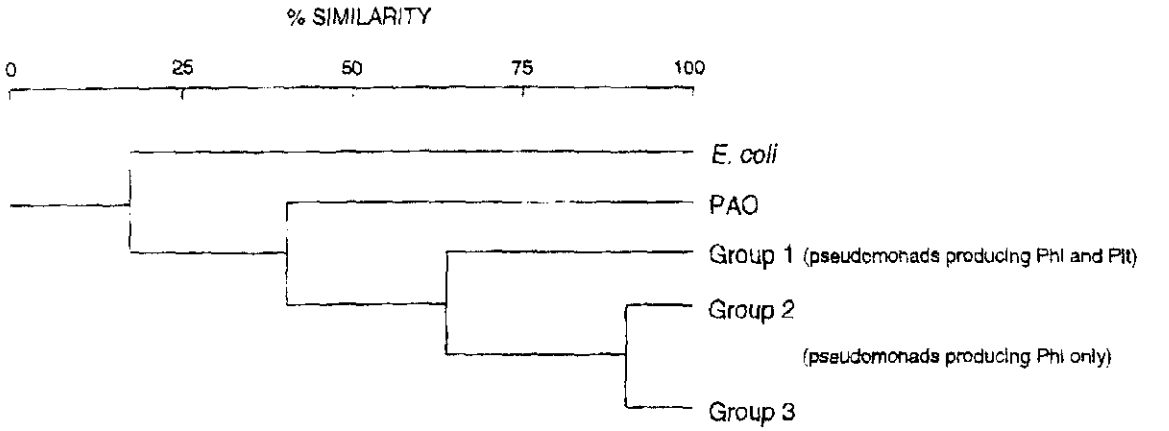

FIG. 2. Cluster dendrogram of PHL-producing *Pseudomonas* strains isolated from different soils worldwide, based on ARDRA. ARDRA was performed with eight restriction endonucleases. The pairwise coefficients of similarity (Jaccard) were clustered by using the UPGMA algorithm of NTSYS-pc. ARDRA group 1 includes all the strains from Switzerland, the United States, Italy, and Ghana, producing both PHL and PLT; ARDRA group 2 contains all the other strains from Swiss, U.S., and Italian soils that produced PHL but not PLT, with the exception of strains P12 and F113, which are contained within ARDRA group 3 (Tables 4 and 5). *E. coli* K-12 and *P. aeruginosa* PAO1 were used as controls.

group contained strains PIHL1, PITR2, and PITR3 from the Italian soil (Fig. 4; Table 4). Strains F113 and P12, which formed ARDRA group 3, gave completely different RAPD fingerprints from each other and from the other strains with each of the four primers used for analysis (data not shown).

DISCUSSION

Production of PHL has been shown to be an important mechanism of biological control of a wide variety of plant pathogens by fluorescent *Pseudomonas* spp. (11, 16, 17, 23, 25, 45, 55). This report is the first to compare PHL production in a worldwide collection of PHL-producing *Pseudomonas* spp. originating from Switzerland, the United States, Ireland, Italy, and Ghana. We conclude that the PHL biosynthetic region is conserved among producer strains because a 4.8-kb chromosomal DNA region from strain Q2-87, encoding unique DNA sequences consistent with synthesis of PHL (2, 3, 16, 55), hybridized under conditions of high stringency with genomic DNA of all 45 PHL-producing strains. This conclusion is further supported by a subsequent survey (26), which demonstrated a relationship between PHL production and hybridization with the 4.8-kb probe in pseudomonads from soils of the Czech Republic, Slovakia, Romania, China, India, and Australia.

Strains in the collection, although having common PHL biosynthetic genes, displayed considerable phenotypic and genotypic diversity. For example, the amount of PHL produced in vitro on malt agar and KMB differed by up to 49-fold and 25-fold, respectively. Whether the different levels of antibiotic production may also influence the biocontrol activity of these strains in the natural environment remains to be tested. Furthermore, on the basis of the metabolites that were assayed, including PHL, PLT, and HCN (all known to be involved in biocontrol activity), two phenotypically distinct groups of PHL producers were apparent. The first group produced PHL, HCN, and PLT and consisted of 13 strains from all of the locations sampled except Ireland. The second group, consisting of 32 strains from the United States, Switzerland, Italy, and Ireland, produced only PHL and HCN. RFLP, ARDRA, and PCR-RAPD analysis indicated that the strains that produced both PHL and PLT were, in fact, genetically similar. For ex-

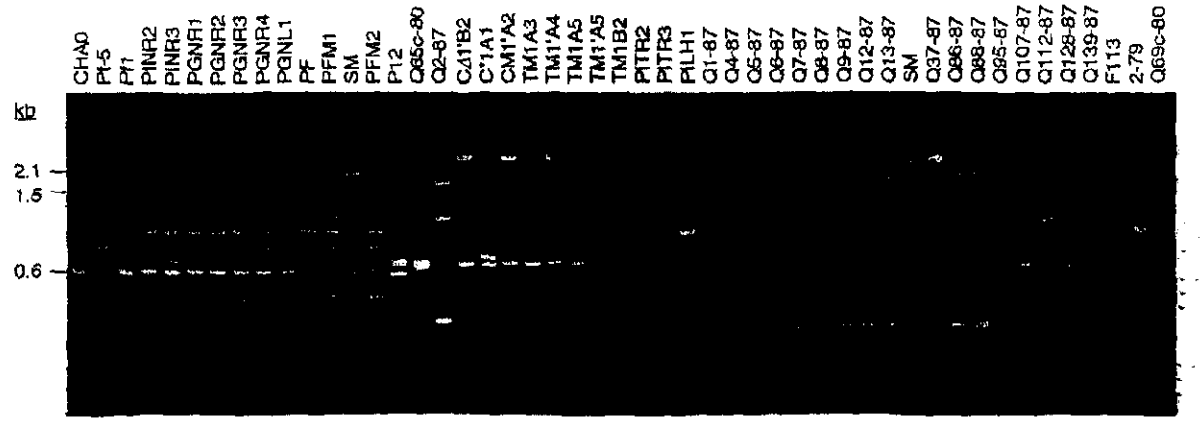

FIG. 3. RAPD products for primer D7 of PHL-producing *Pseudomonas* strains isolated from different soils worldwide. SM, size marker (100-bp ladder [GIBCO]). The naturally PHL-negative strains 2-79 and Q69c-80 were used as controls.

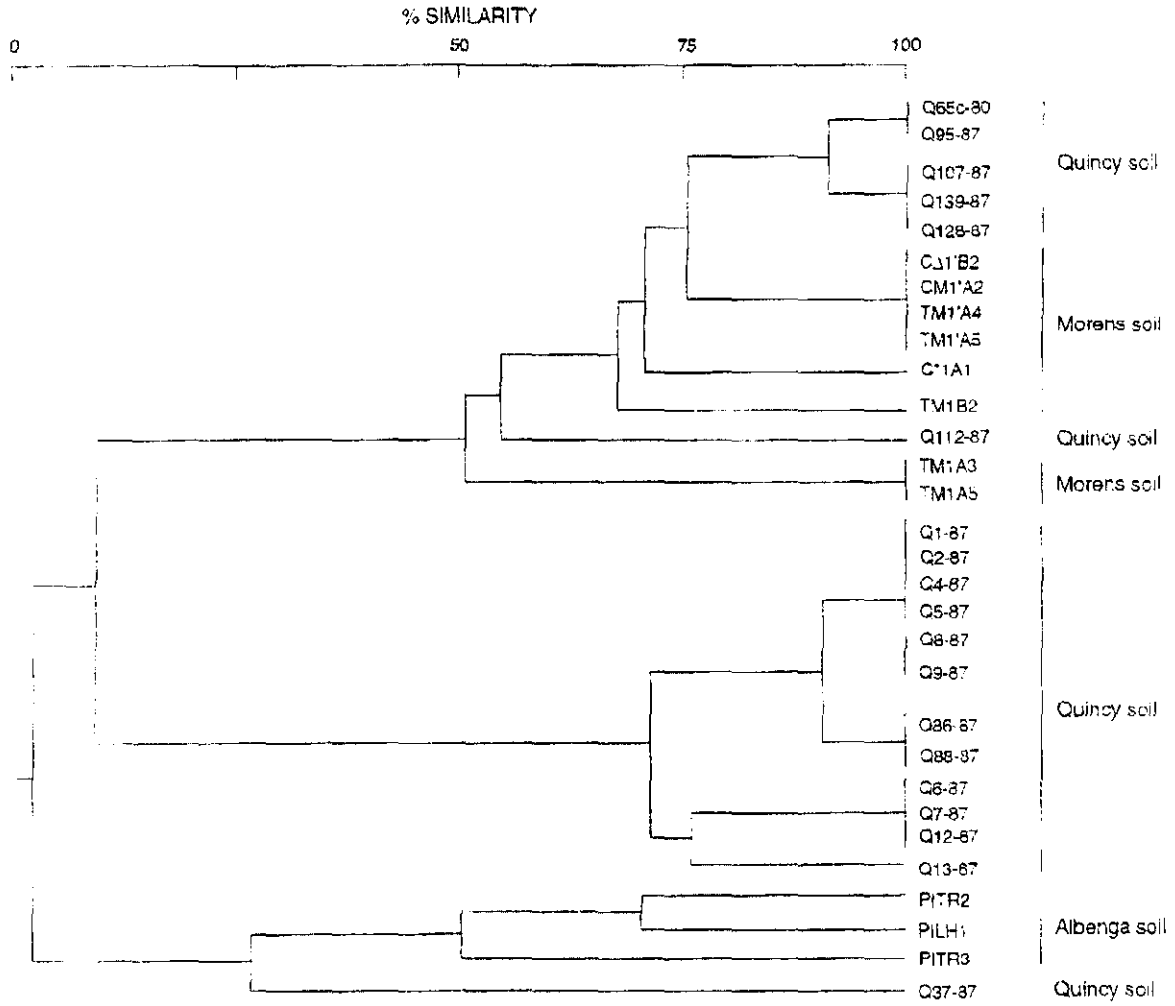

FIG. 4. Cluster dendrogram showing the relationships of PHL-producing *Pseudomonas* strains isolated from different geographic locations worldwide based on RAPD markers. RAPD analysis was performed with four primers, and 14 markers were evaluated. The pairwise coefficients of similarity (Jaccard) were clustered by using the UPGMA algorithm of NTSYS-pc. Relationships are shown for strains from ARDRA group 2 only, which includes strains producing PHL but not PLT (Fig. 2; Table 3).

ample, cluster analysis of ARDRA products showed that the strains constituted a single group. In contrast, there was more diversity at the genotypic level in the strains that produced PHL but not PLT. ARDRA indicated two clusters (groups 2 and 3), and on the basis of RAPD analysis, ARDRA group 2 split into three major subgroups that showed additional polymorphism. It is apparent from these studies that there is a fair amount of diversity within the population of PHL producers, which can be exploited in future screening programs for biocontrol agents. Perhaps most interesting are the observations that the occurrence of most groups of phenotypically and genotypically similar PHL producers was not associated with a specific geographic origin and that highly similar strains could be isolated from diverse locations worldwide. A similar observation recently was reported by Stabb et al. (46), who showed that producers of the aminopolyol antibiotic zwittermicin A made up roughly 9% of the culturable *Bacillus cereus* soil population and were present in many soils from Panama, Honduras, the United States, The Netherlands, and Australia. The finding that *Pseudomonas* strains of different geographic origins commonly produce the same antibiotic(s) and are highly similar genetically raises the questions of whether such strains can perform in locations other than those from which they were isolated and whether additional physiological adaptations to a specific crop and local soils are necessary. If the latter proves to be the case, the availability of genetic probes for important biocontrol traits such as PHL production should greatly facilitate the identification of effective and locally adapted biocontrol agents.

Suppressive soils are "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (1). Given that PHL producers were readily isolated from natural suppressive soils, we speculate that PHL-producing strains may represent a significant fraction of root-associated pseudomonads and hence may play a role in the natural biological control that occurs in some such soils. For example, at least 20% of the fluorescent *Pseudomonas* strains isolated from wheat roots grown in the take-all-suppressive soil from

Quincy had phenotypes typical of PHL producers (17). Furthermore, colony hybridization tests with the 4.8-kb *phl* gene probe with more than 1,100 *Pseudomonas* isolates from wheat roots grown in *T. basicola*-suppressive soils from the Morens region in Switzerland demonstrated that up to 23% contained PHL biosynthetic genes (26).

Despite the interest worldwide in the use of root-associated bacteria (rhizobacteria) as delivery systems for gene products critical to plant protection and although biological control is recognized as the best alternative to the use of chemical pesticides for disease control, there is some concern about the possible adverse nontarget effects of introducing large populations of biocontrol agents into an agroecosystem. The concern is greater when the agents are nonindigenous or genetically engineered. However, our finding that important biocontrol genes, e.g., those involved in the production of PHL, or biocontrol traits, e.g., production of PLT and HCN, already are broadly distributed in root-associated pseudomonads in soils on different continents should lessen concerns about the release of either nonindigenous agents containing these traits or transgenic biocontrol agents into which these traits have been introduced and may speak for a freer exchange of microbial germplasm between countries.

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